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Toxicological assessment of 4-hydroxycoumarin: (Anti) Mutagenicity, toxic, and antioxidant studies

Avaliação toxicológica da 4-hidroxicumarina: (Anti) Mutagenicidade, estudos de toxicidade e antioxidantes

Evaluación toxicológica de la 4-hidroxicumarina: Estudios (anti) mutagénicos, tóxicos y antioxidantes

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Abstract

4-hydroxycoumarin (4H) is a natural and synthetic phenolic compound that presents important attributes, including anti-HIV, antitumor, and anticoagulant properties. Considering its important biological activities and the use of 4H, the aim of this present study was to investigate the toxic, mutagenic, antimutagenic, and antioxidant activities of 4H, commercially obtained, in experiments *in vivo* and *in vitro*. The effects of acute oral administration at a dose of 200 mg/kg of 4H were investigated by the hippocratic screening in animals. The micronucleus assay on peripheral blood and the Ames test were used for analysis of mutagenicity, while the antimutagenicity was tested against cyclophosphamide also by *in vivo* micronucleus assay. For all *in vivo* tests were used Swiss mice. The antioxidant activity was determined by the capacity of the 4H sample to sequester the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The results showed that 4H no induced changes in the behavior or the histology of treated animals neither

mutagenic activity (genic or chromosomal). On the other hand, it exhibited a protective effect against cyclophosphamide and antioxidant potential. 4- hydroxycoumarin is widely used clinically as a precursor molecule in the synthesis of synthetic anticoagulant drugs. Therefore, the results obtained are extremely important, as they demonstrate the absence of mutagenic potential and protection against chromosomal damage.
Keywords: Antioxidants; Coumarins; Genotoxicity; Micronucleus tests; Mutagenicity Tests.

Resumo

A 4-hidroxicumarina (4H) é um composto fenólico natural e sintético que apresenta atributos importantes, incluindo propriedades anti-HIV, antitumoral e anticoagulante. Considerando suas importantes atividades biológicas e a utilização da 4H, o objetivo do presente estudo foi investigar as atividades tóxica, mutagênica, antimutagênica e antioxidante da 4H, obtido comercialmente, em experimentos *in vivo* e *in vitro*. Os efeitos da administração oral aguda na dose de 200 mg / kg de 4H foram investigados pela triagem hipocrática em animais. O ensaio de micronúcleo em sangue periférico e o teste de Ames foram utilizados para análise de mutagenicidade, enquanto a antimutagenicidade foi testada contra a ciclofosfamida também pelo ensaio de micronúcleo *in vivo*. Para todos os testes *in vivo* foram utilizados camundongos Swiss. A atividade antioxidante foi determinada pela capacidade da amostra 4H de sequestrar o radical estável 2,2-difenil-1-picrilhidrazil (DPPH). Os resultados mostraram que o 4H não induziu alterações no comportamento ou na histologia dos animais tratados nem na atividade mutagênica (gênica ou cromossômica). Por outro lado, exibiu efeito protetor contra ciclofosfamida e potencial antioxidante. A 4H é amplamente utilizada clinicamente como uma molécula precursora na síntese de fármacos anticoagulantes sintéticos. Portanto, os resultados obtidos são de extrema importância, pois demonstram ausência de potencial mutagênico e proteção contra danos cromossômicos.
Palavras-chave: Antioxidante; Cumarinas; Genotoxicidade; Teste de Mutagenicidade; Teste para Micronúcleo.

Resumen

La 4-hidroxicumarina (4H) es un compuesto fenólico natural y sintético que presenta importantes atributos, que incluyen propiedades anti-VIH, antitumorales y anticoagulantes. Considerando sus importantes actividades biológicas y el uso de 4H, el objetivo de este estudio fue investigar las actividades tóxicas, mutagénicas, antimutagénicas y antioxidantes de 4H, obtenidas comercialmente, en experimentos *in vivo* e *in vitro*. Los efectos de la administración oral aguda a una dosis de 200 mg / kg de 4H se investigaron mediante el cribado hipocrático en animales. El ensayo de micronúcleos en sangre periférica y la prueba de Ames se utilizaron para el análisis de mutagenicidad, mientras que la antimutagenicidad se probó contra ciclofosfamida también mediante ensayo de micronúcleos *in vivo*. Para todas las pruebas *in vivo* se utilizaron ratones suizos. La actividad antioxidante se determinó por la capacidad de la muestra de 4H para secuestrar el radical estable 2,2-difenil-1-picrilhidrazilo (DPPH). Los resultados mostraron que 4H no indujo cambios en el comportamiento o la histología de los animales tratados ni actividad mutagénica (génica o cromosómica). Por otro lado, exhibió un efecto protector contra la ciclofosfamida y potencial antioxidante. La 4H se utiliza ampliamente en la clínica como molécula precursora en la síntesis de fármacos anticoagulantes sintéticos. Por tanto, los resultados obtenidos son de suma importancia, ya que demuestran la ausencia de potencial mutagénico y protección frente al daño cromosómico.
Palabras clave: Antioxidante; Cumarinas; Genotoxicidad; Pruebas de micronucleus; Pruebas de Mutagenicidad.

1. Introduction

The heterocyclic compounds are important, as they present in natural and synthetic products, and have several biological activities (Clardy & Walsh, 2004). Coumarins and their derivatives are commonly found in several natural and pharmaceutical products (Fylaktakidou et al., 2004). 4-hydroxycoumarin is a direct synthetic precursor to commonly used oral anticoagulants (e.g., warfarin) and rodenticides(Shen et al., 2017). Among various coumarin derivatives, the 4H derivatives are of great importance as they present in many natural products and show a wide range of biological activities such as anti-HIV (Thaisrivongs et al., 1996), anticancer (Stanchev et al., 2008), anticoagulant (Abdelhafez et al., 2010), and antioxidant (O'Reilly, 1976).

Most chronic and incurable diseases are the results of an overload of free radicals that attack the human system, leading to a similar condition as oxidative stress, furthermore protein and DNA-like macromolecules are vulnerable targets for free radical attacks (Evans & Cooke, 2004). The phenolic structure of coumarin can inhibit the formation of reactive oxygen species, such as hydroxyl radical and hydrogen peroxide, and therefore, coumarin derivatives can be potent inhibitors of reactive oxygen species (Sharma et al., 2005).

The evaluation of coumarin derivatives such as 6,7 dihydroxycoumarin (aesculetin) (Souza et al., 2015), a natural and synthetic coumarin derivative, and the evaluation of 4-methylsculetin (Fedato & Maistro, 2014), a synthetic coumarin derivative, exhibited, by toxicological testing, neither toxic nor mutagenic potential. Nonetheless, Jaramillo-García et al., (2018), isolated coumarins from *Baccharis trinervis* leaves used in traditional medicine, and found that coumarins might be responsible for toxic, genotoxic, and mutagenic effects found through the Ames and Micronucleus Tests.

Due to the bioactive potential, widespread use of 4H and contradictory reviews of the literature on coumarin toxicity and lack of specific 4H data, the objective of this work was to evaluate the toxic, mutagenic, antimutagenic and antioxidant potential of commercial 4H sample.

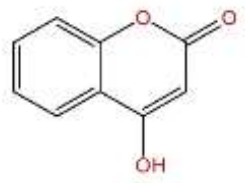
2. Methodology

This is an experimental research, carried out in a laboratory, with a quantitative approach (Pereira et al., 2018) that aimed to evaluate the toxic, mutagenic, antimutagenic and antioxidant potential of commercial 4H sample, through the acute oral toxicity test, the Ames, Micronucleus test and the antioxidant potential test. According to Silva et al., (2014), quantitative research is carried out through data collection through the use of measurements of values, the measurement made through numbers with their respective units. Therefore, the application of this method is necessary to verify the results obtained from the objectives proposed in the study.

2.1 Chemicals

Commercial 4H (Figure 1), dimethyl sulfoxide (DMSO), and the standard mutagenic agents used as positive controls in the Ames Test, these being, 4-nitro-o-phenylenediamine, sodium azide, mitomycin C, 2-aminoanthracene, and 2-aminofluorene, 2,6, as well the positive controls in the antioxidante test, di-tert-butyl-4 -methylphenol (BHT), and 2,2-diphenyl-1-picrylhydrazyla (DPPH) were obtained from Sigma-Aldrich® Brazil. Cyclophosphamide chemotherapy (CP), which was used as the chromosomal damage-inducing agent, was obtained commercially from Genuxal®, Brazil.

Figure 1: Chemical structure of 4-hydroxycoumarin.



Source: Authors.

In Figure 1, we can see 4-hydroxycoumarin, which is considered a simple coumarin derivative, containing a hydroxy radical at the 4-position of coumarin per se (1,2-benzopyrone).

2.2 Animals and Ethics

The *in vivo* studies were carried out using Swiss male mice (Mus musculus) weighing between 30-35 g and 8 weeks old, obtained from the Central Animal Facility of Federal University of São João del-Rei (UFSJ). The animals were kept in the animal experimentation room of the Pharmacology Laboratory of the Center West Campus of the UFSJ (Room 204, block E) under controlled temperature conditions (23oC ± 1oC), with a 12-hour light-dark cycle, and kept in plastic-lined boxes with free

access to food and water. The experimental protocols were approved by the Animal Use Ethics Committee of UFSJ (protocol nº 034/2016 and 002/2018).

2.3 Acute oral toxicity assay

The acute oral toxicity assessment of 4H was performed based on the method recommended by the Organization for Economic Cooperation and Development guidelines for the test of acute oral toxicity (Guideline 425) with minor modifications (Botham, 2004; Guideline, 2001; Mythilypriya et al., 2007). The mice were randomly divided into six groups (n=6). Group 1 animals received distilled water only; Group 2 animals received 20 % DMSO in saline 0.9 % (solvent used to prepare 4H solutions), and Groups 3, 4 and 5 were treated with a solution at doses of 50, 100, 200 mg/kg body weight (b.w.) respectively of 4H. The animals received 0.3 mL of each treatment orally.

The animals were continuously observed at 10, 30, and 60 min after the administration of treatments to evaluate any changes in autonomy, behavioral, and general neurological profiles. Subsequently, the animals were observed periodically at 2, 4, 8, and 12 h after treatment administration, and then once a day for 14 days. The five parameters of hippocratic screening were analyzed (Cheng, 2016).

For verification of tissue toxicity reactions, the animals were euthanized on the 15th day after administration, and the organs (heart, lung, spleen, liver, and kidney) were removed, weighed, and macroscopically examined. The liver obtained from each animal was studied microscopically to evaluate and determine possible aggressions to the organ.

2.4 Ames test

Four histidine-dependent stains of *Salmonella typhimurium* (TA98, TA100, TA102, and TA97a) were used for the bacterial reverse mutation assay, and the experiment was performed according to the guidelines recommended by Maron and Ames (Maron & Ames, 1983).

The concentrations were selected based on preliminary toxicity tests and ranged from 6.25 to 50.0 µg/plate. The S9 fraction was purchased from Molecular Toxicology Inc. (Boone, NC, USA) (Maron & Ames, 1983). All assays were conducted in triplicate. The mutagenicity index (MI) was calculated by the number of revertants in the test compound plate (induced revertant) divided by the number of revertants in the negative control (solvent) plate for each concentration tested. The sample was considered mutagenic when the MI is greater than or equal to two in at least one of the tested doses (Mortelmans & Zeiger, 2000).

2.5 Micronucleus Test

The animals were divided into nine experimental groups of six animals each. For mutagenicity evaluation, the animals were divided into a negative control group (treated with water orally), positive control group (treated with CP 50 mg/kg b.w., ip), solvent control group (treated with 20% DMSO saline solution orally), and three treatment groups treated with 4H orally at concentrations of 50, 100, and 200 mg/kg b.w.(MacGregor et al., 1980). For the evaluation of antimutagenicity, the groups treated with 4H were associated with CP (Hayashi, 2016), at the same concentrations used in mutagenicity tests solvent solution plus CP was also evaluated. Peripheral blood samples were collected between 18 and 24 hours after treatment (Furtado et al., 2008).

To evaluate the percentage reduction in the frequency of micronucleated polychromatic erythrocytes (PCEMN), a total of 12000 PCE (polychromatic erythrocytes), were analyzed in each group was calculated by the number of PCEMN obtained after CP treatment minus the number found with antimutagenic treatments (4H + CP) divided by the number of PCEMN observed

with CP minus the number obtained for the negative control. To evaluate the cytotoxicity, 400 total peripheral blood erythrocytes were analyzed per animal, obtaining the ratio PCEs/PCEs + NCEs [normochromatic erythrocytes], in the different treatments (Furtado et al., 2008).

2.6 Antiradical activity: DPPH˙ assay

The antioxidant potential of 4H was analyzed compared to standard 2,6-di-tert-butyl-4-methylphenol (BHT) based on the reaction with 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). The determination of antioxidant activity using the DPPH method was adapted for use with microplates (Araújo et al., 2013).

Briefly, a DPPH solution (0.002% w/v) was prepared in ethanol. Exactly 75 µL of the 4H samples (1, 10, 100, 250, and 500 µg/mL) were added to the wells of a 96-well flat-bottom plate containing 150 µL of DPPH solution. The plate was then covered and left in the dark at room temperature (25°C). After 30 min, the absorbance at 517 nm was measured with a spectrophotometer (Biotek Power Wave XS2, USA), and ethanol was used for the baseline correction. Scavenging ability was expressed as the inhibition percentage and was calculated using the following equation (Gülçin, 2006): Scavenging ability = {(Abs Control-Abs sample)/Abs control} x 100, where Abs control = absorbance of DPPH radical in ethanol and Abs sample = absorbance of the 4H or BHT in ethanol + DPPH.

The antioxidant activity of all of the samples was expressed as IC50, which was defined as the concentration (in µg/mL) of sample required to inhibit the formation of the DPPH radicals by 50%. All assays were performed in triplicate.

2.7 Statistical analysis

The results obtained from the Ames Test were analyzed using the Salanal statistical program (US Environmental Protection Agency, Monitoring Systems Laboratory, Las Vegas, NV, version 1.0, Research Triangle Institute, RTP, North Carolina, USA), using the Bernstein model (Bernstein et al., 1982). Data (revertant/plate) were evaluated by analysis of variance (ANOVA), followed by linear regression.

The other results were statistically assessed by ANOVA for entirely randomized experiments, with the calculation of the F statistic and its respective p-value. In cases $p < 0.05$, the means were compared by the Tukey test, with the calculation of the minimum difference for $\alpha = 0.05$.

3. Results

3.1 Oral Toxicity

According to the evaluation of acute oral toxicity of 4H, the results showed that the dose tested produced no visible signs or symptoms of toxicity in the mice tested as there was no difference in behaviors observed between the 4H-treated animals and the negative controls. There were also no deaths within 14 days after administration. There was no statistically significant difference between control and treatment groups for water and feed intake. The organs analyzed (heart, lung, spleen, liver, and kidney) were macroscopically unchanged, with normal architecture, and no necrosis or hemorrhagic areas. The relative mass of these organs in all animals displayed no difference between groups, as shown in Table 1.

Table 1. The relative mass of organs after euthanasia of mice treated with 4-hydroxycoumarin (4H) at a single dose of 200 mg/kg.

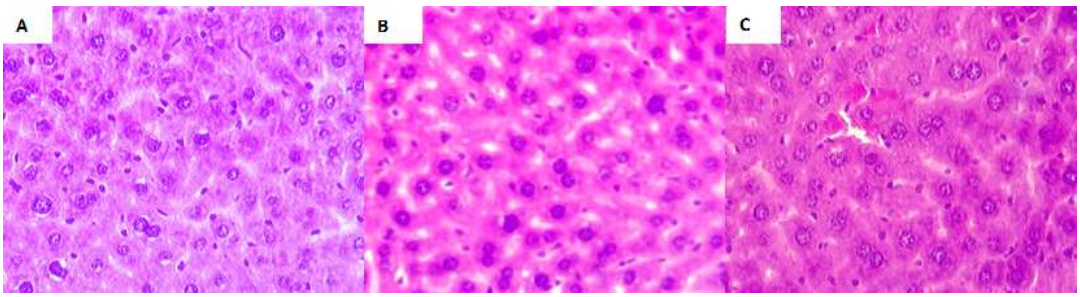
Organs (g)	Negative Control Mean ± SD	Solvent Control Mean ± SD	4H Mean ± SD
Heart	5.25 ± 0.11	5.23 ± 0.06	5.27 ± 0.09
Lung	6.88 ± 0.12	6.81 ± 0.08	7.24 ± 0.03
Spleen	4.49 ± 0.06	5.29 ± 0.16	5.93 ± 0.12
Right Kidney	8.07 ± 0.09	7.02 ± 0.04	7.73 ± 0.08
Left Kidney	7.94 ± 0.08	7.83 ± 0.04	8.06 ± 0.03
Liver (g/100g live weight)	6.67 ± 0.60	6.40 ± 0.40	6.87 ± 0.72

M ± SD = mean and standard deviation. The negative control was administered water, and the solvent control group was administered 20% DMSO in saline.
Source: Authors.

Table 1 shows the relative mass of organs (heart, lung, spleen, liver and kidney) after euthanasia of animals treated with 4-hydroxycoumarin in a single dose of 200 mg/kg. The negative control was administered with water and the solvent control group was administered with 20% DMSO in saline. We can observe that there was no statistical difference in the relative mass of the organs between the treatment group (4H) and the control groups.

Histopathological analysis of the livers after 15 days of administration of the controls and treatments showed normal preserved liver architecture with the disposition of the hepatic tissue in lobes, central lobular vein, where the hepatic sinusoids, intact hepatocytes, and large nuclei (Figure 2).

Figure 2. Photomicrographs of histological sections of hematoxylin-eosin (H.E.) stained livers, 200x magnification, 15 days after compound administration.



Negative control (A), Solvent control (B), 4H 200 mg/kg BW (C). Source: Authors.

Figure 1 shows the photomicrographs of histological sections of the liver and demonstrates that the liver architecture was preserved, and was within normal limits, with intact hepatocytes 15 days after administration of both controls and treatment with 4H.

3.2 Ames Test

Ames test results were expressed by the mean of the number of revertant/plate (M), standard deviation (SD), and mutagenicity index (MI) after treatments at different 4H concentrations, observed in the strains TA98, TA100, TA97a and TA102 of *S. Typhymurium* in the presence (+S9) and absence (-S9) of metabolic activation are shown in Table 2. After analyzing the results, it was observed that 4-H was not able to induce gene mutations by Ames test.

Table 2. Mutagenic activity expressed by means and standard deviation of the number of revertants / plate and mutagenicity index in the TA97a, TA98, TA100 and TA102 strains of *S. Typhimurium* after treatment with various concentrations of 4-hydroxycoumarin (4H), in experiments without (-S9) and with (+ S9) metabolic activation.

	Number of revertants (M ± SD)/plate and MI							
Treatments	**TA97a**		**TA98**		**TA100**		**TA102**	
µg/ plate	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
0[a]	164 ± 21	154 ± 32	15 ± 4	19 ± 3	124 ± 13	103 ± 10	221 ± 37	244 ± 41
6.25	210 ± 27 (1.28)	204 ± 10 (1.33)	14 ± 1 (0.97)	20 ± 2 (1.05)	148 ± 28 (1.20)	117 ± 12 (1.14)	310 ± 43 (1.40)	261 ± 21 (1.07)
12.5	223 ± 19 (1.36)	219 ± 21 (1.42)	14 ± 3 (0.97)	21 ± 1 (1.11)	138 ± 10 (1.12)	114 ± 17 (1.11)	337 ± 24* (1.52)	267 ± 19 (1.09)
25	230 ± 28 (1.40)	182 ± 31 (1.19)	18 ± 2 (1.24)	22 ± 4 (1.16)	150 ± 19 (1.21)	130 ± 14 (1.26)	335 ± 39* (1.51)	292 ± 12 (1.19)
37.5	241 ± 35 (1.47)	190 ± 14 (1.23)	17 ± 2 (1.17)	20 ± 3 (1.05)	145 ± 24 (1.17)	104 ± 22 (1.00)	261 ± 37 (1.18)	294 ± 24 (1.20)
50	214 ± 17 (1.30)	187 ± 19 (1.21)	12 ± 1 (0.83)	17 ± 2 (0.89)	147 ± 21 (1.19)	121 ± 18 (1.17)	255 ± 30 (1.15)	262 ± 10 (1.07)
C+	1473 ± 71[b]	1851 ± 116[e]	791 ± 24[b]	842 ± 32[e]	2231 ± 114[c]	1134 ± 157 [e]	2240 ± 151[d]	2430 ± 237[f]

Source: Authors.

Table 2 demonstrates that 4H was not considered mutagenic as it did not induce a statistically significant increase in the number of revertants when compared to the negative control, nor did it induce an MI greater than 2.0, both in the presence (+S9) and absence (-S9) of metabolic activation of the Ames test, indicating absence of direct mutagenic activity.

3.3 Micronucleus Test

Mutagenic effects were assessed from the micronucleus test. Table 3 summarizes the frequency of PCEMN and the PCE/NCE rate in peripheral blood of mice treated with different doses of 4H. The results revealed that 4H does not induce chromosomal mutation since no doses tested caused an increase in the frequency of PCEMN. In addition, the groups in which 4H was associated with CP, a considerable protective effect was seen, since the dose of 200 mg/kg BW presented a reduction of approximately 96% in PCEMN, demonstrating its antimutagenic effect. Regarding the cytotoxic capacity of 4H, it was observed that non-cytotoxic 4H is able to attenuate the cytotoxic action of CP at all doses tested.

Table 3. Frequency of micronucleated polychromatic erythrocytes (PCEMN) and peripheral blood cytotoxicity of 4-hydroxycoumarin (4H) and cyclophosphamide (CP) treated Swiss mice with their respective controls.

Treatments	**PCE/PCE+NCE**	**PCEMN**		**Reduction**
	Mean ± SD	**Number**	**%**	**%**
Control [b]	0.04 ± 0.004	44	0.36	-
DMSO [b]	0.04 ± 0.003	34	0.28	-
4H 50 [b]	0.04 ± 0.006	51	0.42	-
4H 100 [b]	0.04 ± 0.006	50	0.41	-
4H 200 [b]	0.04 ± 0.006	55	0.45	-
CP [a]	0.04 ± 0.004	225	1.87	-
DMSO + CP [a]	0.04 ± 0.003	217	1.8	-
4H 50 + CP [a b]	0.04 ± 0.006	97	0.8	70.71
4H 100 + CP [b]	0.04 ± 0.006	55	0.45	93.92
4H 200 + CP [b]	0.04 ± 0.005	51	0.42	96.13

A total of 12000 PCE were analyzed in each group. [a]Significantly different from the negative control ($p < 0.05$). [b]Significantly different from the CP group ($p < 0.05$). Source: Authors.

In Table 3, we can see the frequencies of PCEMNs in the peripheral blood of Swiss mice treated with 4H and controls alone or in combination with cyclophosphamide. The table presents the raw and percentage values of PCEMNs, as well as the percentage of reduction in the frequency of PCEMNs. The frequency of PCEMNs increased in animals that received only CP (positive control), presenting a statistically significant difference compared to animals that received only water (negative control). Furthermore, there was no significant difference between animals from the negative control and the animals that received DMSO + 20% saline (solvent control), demonstrating the absence of a mutagenic effect of the solvent. The treatments with 4H, at the different doses tested, did not show an increase in the frequency of PCEMNs when compared to the negative control, which indicates the absence of a mutagenic effect under the conditions used in this study. In evaluating the antimutagenic effect, the treatment with DMSO + 20% saline associated with CP demonstrated that the solvent does not exert a protective effect against chromosomal damage, presenting a frequency of PCEMNs similar to that induced by the positive control. However, the concomitant administration of 4H and CP resulted in a reduction in the frequency of PCEMNs when compared to the group treated only with the chemotherapy

3.4 Antiradical activity

Analysis of the antioxidant activity of 4H through DPPH radical scavenging compared to the BHT positive control, and IC50 values are shown in Table 4.

Table 4. DPPH scavenging activity and IC_{50} values for antioxidant activity of of 4 hydroxycoumarin (4H).

Sample	DPPH scavenging activity (%)					IC_{50}
	1 μg/mL	10 μg/mL	100 μg/mL	250 μg/mL	500 μg/mL	
4H	33.87 ± 0.29[a]	34.53 ± 0.22[a]	36.05 ± 0.79[a]	38.31 ± 0.51[a]	54.29 ± 1.31[a]	439.52 ± 35.70
BHT	18.50 ± 0.65	25.90 ± 0.64	86.00 ± 0.56	91.40 ± 0.28	94.02 ± 0.51	16.36 ± 1.63

DPPH: Di-tert-butyl-4–methylphenol; BHT: Di-tert-butyl-4–methylphenol; IC_{50}: concentration (in μg/mL) of samples required to inhibit the formation of DPPH radicals by 50%. Each value in the table is the mean ± standard deviation (n=3). [a]$p < 0.05$ compared with BHT. Source: Authors.

In Table 4, it can be seen that 4H inhibited the DPPH radical and had its best performance at the concentration of 500 μg / mL where it reduced the free radical by half. However, even at the lowest concentrations (10 and 1 μg / mL), the 4H samples showed a better percentage of antioxidants when compared to the BHT control.

4. Discussion

The balance between the therapeutic and toxicological effects of a drug is an important parameter in the evaluation of its applicability concerning pharmacological action (Ezuruike & Prieto, 2014).

Studies show that no single test can detect the full spectrum of different endpoints covered by induced genotoxicity or to identify various potential protection mechanisms for genetic material (antigenotoxicity) (Dearfield et al., 2002; Sun et al., 2020; Zendeboodi et al., 2020). For this reason, in the present work, we combined the *in vivo* acute oral toxicity test, Ames test, the *in vivo* Micronucleus test, and, finally, the *in vitro* test of 4H antioxidant activity.

In all trials, the measured endpoints were different. The evaluation of subacute oral toxicity determines if the effective dose for biological activities can cause tissue and organ toxicity. Furthermore, this test allows us to verify the form of adverse reactions that test compounds cause in target organs and behavioral changes. It also provides information for risk assessment of acute exposure, verifies the late effects of treatment, and if there is the reversibility of toxic response (Porwal et al., 2017).

The acute oral LD50 of coumarin has been reported to range from 196 to 780 mg/kg in various mouse strains (Egan et al., 1990; Lake, 1999). Specifically, the LD50 of 4H was not demonstrated. On the other hand, some studies have shown that 4H was non-toxic to laboratory animals in acute or chronic oral gavage administration at doses equal to or higher than 200 mg/kg (Maryna L. Zinovieva & Peter G. Zhminko, 2017; Vasconcelos et al., 2009). We did not observe any signs of toxicity in 4H - treated mice in our study.

In the assessment of acute oral toxicity presented in this study, the animals were treated with a single oral dose of 200 mg/kg of 4H, as this was the maximum dose used in mutagenicity studies. At 14 days of observation, there were no signs of adverse effects observed through hippocratic screening.

Acute oral toxicity studies using coumarin derivatives have already been performed, such as tests with umbelliferone (7-hydroxycoumarin associated with the β-D galactopyranoside group) isolated from the stem bark of *Aegle marmelos* (Rutaceae) found throughout India at 10, 20, and 40 mg/kg which revealed its non-toxic nature, as there was no lethality or toxic reaction found in any of the selected doses until the conclusion of the study period (Kumar et al., 2013). S

Stanchev et al., (2008) also indicated that six synthesized 4H derivatives revealed by the oral toxicity test showed different levels of toxicity. However, those closest to the original molecule presented a lower level of toxicity.

In the Ames test, the classical set of *Salmonella* formed by the strains TA98, TA100, TA102, and TA97a for mutagenicity tests permits the detection of a wide range of damages, from frameshift to basepair mutations caused by small DNA adducts formation and intercalation into the DNA. The test, also called a bacterial reversal, occurs because of new mutations at or near pre-existing mutated genes that restore gene function and allow bacteria to synthesize the amino acid histidine necessary for their growth. The test is used as a screen to determine the mutagenic potential, because of the high predictive value for rodent carcinogenicity. In addition, the exogenous mammalian metabolic activation system (S9) was used to simulate the metabolization of the samples, once the bacteria are unable to metabolize chemicals via cytochromes P450, as in mammals and other vertebrates (Mortelmans & Zeiger, 2000).

In vivo tests and the *Salmonella*/microsome assay are the most frequently used and recommended by regulatory agencies for determining genetic risk (FDA, 2012), since the *Salmonella*/microsome assay has a high predictive value for carcinogenicity in rodents when a mutagenic response is obtained (Mortelmans & Zeiger, 2000) and they also take into account factors regarding *in vivo* metabolism, pharmacokinetics and DNA repair processes (Krishna & Hayashi, 2000; Tsuboy et al., 2010). In our study, 4H was non-mutagenic with and without S9 activation.

Finn et al., (2002) have also shown the absence of mutagenicity of coumarin derivatives such as 6,7-hydroxycoumarin and 4-methylsculetin. Similarly, other coumarin studies evaluated the mutagenic potential of eight coumarin compounds through the Ames test, and all were considered non-mutagenic (Maistro et al., 2015).

The ability to monitor micronuclei in peripheral blood rather than in bone marrow has a number of major advantages. Since a small drop of blood provides thousands of scorable cells, repeated samples may be easily obtained from a single animal without the necessity of injuring or killing the animal (Hayashi, 2016; MacGregor et al., 1980). The mouse peripheral blood micronucleus test was used to evaluate the mutagenic potential of 4H due to chromosomal damage, and to evaluate the protective antimutagenic effect against chromosomal (clastogenic and/or aneugenic) damage induced by cyclophosphamide and the cytotoxic effect *in vivo*.

The micronucleus test results in mouse peripheral blood showed that there was no increase in the frequency of micronucleated cells in peripheral blood, showing no potential for chromosomal mutagenicity. The concentrations tested were effective in reducing cyclophosphamide-induced mutagenic effects demonstrating the antigenotoxic effect of 4H. Furthermore, none of the doses tested exhibited cytotoxicity.

Souza Marques et al., (2015), they also evaluated the clastogenic/aneugenic potential of 6,7-dihydroxycoumarin (6,7-HC) (esculetin) through the micronucleus test in animals in which the compound was administered orally at doses of 25, 50, and 500 mg/kg, and showed no chromosomal damage effect, and significantly reduced doxorubicin-induced chromosomal damage. Fedato & Maistro (2014), evaluated the mutagenic potential of metilesculetin (4-ME), a synthetic coumarin derivative, by *in vivo* micronucleus testing and found no increase in micronucleated cells between the negative control and the groups treated with doses of 500, 1000, and 2000 mg/kg of 4-ME, besides intense antimutagenic activity against doxorubicin-induced damage.

The consistency between the results reached using the Ames test and the micronucleus test are very important since these methods are complementary and could reveal genotoxic agents (reading frame deviation and base-pair replacement), chromosome damage (clastogenicity and aneuploigenicity) and antigenotoxic agents (Kirkland et al., 2011).

Currently, the inclusion of chemopreventive agents, either topically or orally as dietary factors, is considered a less toxic and more effective approach to chemoprevention of DNA damage, which could lead to carcinogenesis and many degenerative diseases(Bode & Dong, 2000). Although the mechanisms underlying the protective effect of 4H against CP-induced mutagenicity

is not completely understood, a large number of antioxidants inhibit mutagenic activity, since oxygen-free radical formation is an important factor in inducing mutagenesis and carcinogenesis (Su et al., 2013).

In our paper, the potential antioxidant test with inhibition of the DPPH radical showed an intense antioxidant capacity of 4H. Given this, the protective effect of 4H can be explained mainly by its ability to eliminate the generation of free radicals and reactive oxygen species. This protective activity is explained by the ability of the heterocyclic molecules of coumarins to attenuate the oxidative stress process (Fedato & Maistro, 2014). The hypothesis is also explained by the fact that CP is a synthetic antineoplastic belonging to the class of alkylating agents. This class has the property of becoming a strong electrolyte by forming intermediates with the deoxyribonucleic acid (DNA) molecule resulting in structural damage (Masfria et al., 2017).

5. Conclusion

The biological activity of compounds depends on a complex sum of individual properties, including their structure, affinity for the target site, properties within the biological system, and others. In this study, the focus was on toxicity studies of 4H. These portrayed, mainly, the absence of mutagenic potential by both the micronucleus test and Ames test, and protection against chromosomal damage. Therefore, further studies are needed to better characterize the mechanism of action of 4H, both *in vitro* and *in vivo*, as well as further investigation using different long-term animal tests to assess chronic exposure to 4H to clarify the toxic effects, benefits of the protective properties of this compound and safety of its use in humans.

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